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Serial No.: 10/041,958 Art Unit: 1645

Filed: January 7, 2002 Examiner: Mark Navarro

For: *HUMAN NEUTRALIZING ANTIBODIES AGAINST HEMOLYTIC UREMIC SYNDROME*

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

SUBSTITUTE APPEAL BRIEF

Sir:

In response to the notice of the Board of Patent Appeals and Interferences mailed on October 11, 2005, please substitute this Appeal Brief with the Appeal Brief submitted via facsimile on September 14, 2004.

This is an appeal from the rejection of claims 26-36 in the Office Action mailed April 16, 2004, in the above-identified patent application. A Notice of Appeal with authorization to charge Deposit Account No. 50-3129 in the amount of $165.00, the fee for filing a Notice of Appeal for a small entity, was filed on July 16, 2004. The Commissioner was also authorized to charge the fee in the amount of $165.00 for a small entity, for the filing of Appellants' Brief, to Deposit Account No. 50-3129 on September 14, 2004. It is believed that no additional fee is required with this submission. However,

should a fee be required, the Commissioner is hereby authorized to charge the fee to Deposit Account No. 50-3129.

(1) REAL PARTY IN INTEREST

The real party in interest of this application is the assignee, Trustees of Tufts College, Medford, MA. This product is in clinical trials.

(2) RELATED APPEALS AND INTERFERENCES

There is a related appeal in Serial No: 10/230,614 filed August 29, 2002, which directly affects, which would be directly affected by, or which may have a bearing on the Board's decision in this appeal. A decision was rendered in this case on September 26, 2005. This application is a continuation-in-part of the application in the previous appeal.

(3) STATUS OF CLAIMS

Claims 26-36 are pending, rejected and on appeal. The text of each claim on appeal, as amended, is set forth in the Appendix to this Appeal Brief.

(4) STATUS OF AMENDMENTS

The pending claims were last amended by the Amendment mailed January 9, 2004, and entered upon filing of a Request for Continued Examination on February 4, 2004.

(5) SUMMARY OF CLAIMED SUBJECT MATTER

Claim 26 defines a dosage formulation comprising an effective amount of human or humanized monoclonal antibodies, the antibodies consisting of antibodies neutralizing Shiga like toxin II *in vivo*, wherein the antibodies are specifically reactive with a single subunit of the Shiga like toxin II produced by *Escherichia coli* which causes hemolytic uremic syndrome, to prevent or treat hemolytic uremic syndrome in a human (page 6,

lines 15-16; page 7; page 34, lines 11-22); page 37, lines 3-12; page 38, line 12 to page 46, line 15).

Claim 27 defines the antibodies as human monoclonal antibodies (page 5, lines 12-19). Claim 28 defines the antibodies as produced by recombinant DNA methodology (page 5, lines 19-25). Claim 29 defines the antibodies as chimeric monoclonal antibodies (page 6, lines 1-4; page 15, lines 5-24).

Claim 30 restricts the claimed antibodies to antibodies which bind to the alpha subunit of the Shiga like toxin II (page 7, lines 10-15; page 18, line 9 to page 19, line 6; page 34, lines 11-22; page 38, line 12- page 46, line 15; page 47, lines 3-5).

Claim 33 restricts the claimed antibodies to antibodies which bind to the beta subunit of the Shiga like toxin II (page 7, lines 10-15; page 18, line 9 to page 19, line 6; page 34, lines 11-22; page 38, line 12- page 46, line 15).

Claim 31 defines the dosage formulation as being effective to prevent neurological signs of hemolytic uremic syndrome or lesions, wherein the neurological signs or lesions (page 36, lines 11-14) are selected from the group consisting of bloody diarrhea, acute renal failure (page 20, lines 10-14), cerebral hemorrhaging, bacterial shedding into feces, bacterial lesions, paddling, head-pressing, ataxia, convulsions and wasting (page 36, lines 16-20; page 42, lines 1-5). Claim 32 defines the dosage formulation wherein the antibodies are effective to prolong survival (page 40, lines 20-25; page 43, lines 13-25). Claim 34 defines the dosage formulation as equivalent to 4 ml serum from an animal immunized with Shiga-like toxin II/kg body weight (page 20, line 1). Claim 35 defines the dosage formulation as that which is effective to produce a serum level of anti-Shiga toxin II antibodies of at least 0.5 micrograms/ml (page 43, lines

8-12). Claim 36 defines the dosage formulation as that which is equivalent to a dosage of 3 mg human monoclonal antibody to Shiga-like toxin II administered to a newborn pig (page 36, lines 14-16).

(6) GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The issue presented on appeal is whether claims 26-36 are obvious under 35 U.S.C. 103 over U.S. Patent No. 5,512,28 to Krivan *et al.* ("Krivan ") and Perera, et al., J. Clin.Microbiol. 26(10), 2127-2131 (1988) in view of WO 90/07861 by Queen *et al.* and Engelman *et al.*, Human Hybridomas and Monoclonal Antibodies, NY Plenum Press 1985 pp. 23-27 ("Engelman") and further in view of U.S. Patent No. 6,080,400 to Williams.

(7) GROUPING OF CLAIMS

The claims do not stand or fall together, as discussed in more detail below. There are a number of elements not disclosed by the prior art, in addition to the failure to provide the motivation to select and combine the elements as defined by the independent claims.

Claims 27-29 are drawn to the source of the monoclonal antibodies (claim 27, human; claim 28, recombinant DNA; claim 29, chimeric antibodies).

Claims 30 and 33 are specific to particular subunits of the Shiga like toxin II, subunit A and subunit B.

Claim 31 is drawn to a specific formulation for preventing the neurological signs of HUS - none of the prior art even recognizes this is an issue, much less provides guidance on what would be an effective amount to treat or prevent.

Claims 32, and 34- 36 relate to specific dosages. There is no art disclosing or suggesting the effective dosages.

(7) ARGUMENTS

(a) The Invention

Hemolytic uremic syndrome, or HUS, is a disorder marked by kidney failure, hemolytic anemia, thrombocytopenia (platelet deficiency), coagulation defects, and variable nervous system signs. This disorder is most common in children. It frequently occurs after a gastrointestinal (enteric) infection, often one caused by a specific *E. coli* bacteria (*Escherichia coli* O157:H7). It has also been associated with other enteric infections including Shigella and Salmonella and some non-enteric infections.

HUS, once relatively rare, is increasing in children. It is the most common cause of acute kidney failure in children. Several large outbreaks in 1992 and 1993 were attributed to undercooked hamburger contaminated with *E. coli*. Because of this association, supermarket hamburger has new labeling, and there are new temperature guidelines for hamburger cooked at fast-food chains and restaurants. HUS is less common in adults.

HUS is one of the most common causes of sudden, short-term kidney failure in children. In severe cases, this acute kidney failure may require several sessions of dialysis to take over the kidneys' job of filtering wastes from the blood, but most children recover without permanent damage to their health. Most cases of HUS occur after an infection of the digestive system by *Escherichia coli* bacterium, which is found in contaminated foods like meat, dairy products, and juice. Some people have contracted HUS after swimming in pools or lakes contaminated with feces. The infection of the digestive tract is called

gastroenteritis and may cause the child to vomit and have stomach cramps and bloody diarrhea. Most children who experience gastroenteritis recover fully in 2 or 3 days and do not develop HUS. In a few children, however, HUS develops when the bacteria lodged in the digestive system make toxins that enter the bloodstream and start to destroy red blood cells. Symptoms of HUS may not become apparent until a week after the digestive problems. The child remains pale, tired, and irritable. Other symptoms include small, unexplained bruises or bleeding from the nose or mouth that may occur because the toxins also destroy the platelets, cells that normally help clotting. Urine formation slows because the damaged red blood cells clog the tiny blood vessels in the kidneys, making them work harder to remove wastes and extra fluid from the blood. The body's inability to rid itself of excess fluid and wastes may in turn cause high blood pressure or swelling of the face, hands, feet, or the entire body. This progression to acute kidney failure occurs in about half the cases of HUS.

Bacteria such as *E. coli* vary tremendously in the hosts that they infect and which toxins they produce. Some strains primarily affect cattle, others pigs, still others humans. Results obtained using organisms isolated from humans will be different from results obtained from organisms isolated from cattle or pigs. This is an extremely important point. As is clear from the examples in the specification, there is tremendous variation among *E. coli* that cause disease. The *E. coli* that causes disease in children is not the same *E. coli* that causes disease in calves. In calves, the disease is typically characterized by scours - diarrhea, and is most common in dairy calves that are bottle fed. Most farmers do not treat animals by i.v. injection, but prefer to add treatment to the bottles - hence the emphasis in the prior art on enteral treatments for cattle. However, the reason

these *E. coli* infect calves and not people is because the bacteria cannot infect an animal unless they attach to receptors on the gut lining, and the receptors are species specific. This is explained in the declarations discussed in more detail below. Because the receptors are specific to a particular species, one cannot extrapolate from studies in most animals to efficacy or treatments in humans. Only one animal model, discussed below, a newborn piglet which is deprived of colostrums, can be used as a model for bacteria which infect human, because the bacteria which infect mice, rats, and cows will not bind to the receptors in the human gastrointestinal tract - and *vice versa*.

Only recently has it been known which of the many toxins produced by the various bacteria cause the most serious illness. This is in part due to the lack of an appropriate animal model, discussed below, which could be used to compare the effects of the different toxins, and antibodies specific to the different toxins. Appellants were the first to discover that of the two major toxins produced by *E. coli* implicated in HUS in humans, Shiga-like toxin I and Shiga-like toxin II, it is the latter, Shiga-like toxin II, that causes the worst symptoms of HUS, leading to kidney failure and death. This is important since one can screen bacteria to see which toxins, and in which amounts, are produced. As demonstrated by the examples in appellants' specification, bacteria which produce Shiga-like toxin I ("stx1 "), or a mixture of Shiga-like toxin II ("stx2"), are not as deadly as strains that produce primarily Shiga-like toxin II. However, the studies also show that one can administer antibodies directed to the Shiga-like toxin II, rather than a mixture of antibodies, and selectively prevent the worst symptoms of the disease. Since treatment is primarily supportive, the patients will then recover without the permanent kidney damage and other long term side-effects of the disease.

Appellants have developed a dosage formulation to prevent or treat hemolytic uremic syndrome in a human individual exposed to or infected by *Escherichia coli* producing Shiga-like toxin II, which can be administered by administering intradermally, subcutaneously, intravenously, or intramuscularly, to an individual presenting with bloody diarrhea, diagnosed with infection by *Escherichia coli* producing Shiga-like toxin II, or exposed to an individual infected with or exposed to the same source of infection with *Escherichia coli* producing Shiga-like toxin II, comprising an effective amount of monoclonal human or humanized antibodies consisting of antibodies reactive with a single subunit of Stx2, which neutralize Stx2, to prevent or treat hemolytic uremic syndrome in a human (page 5, line 4-page 6, line 11; page 7, lines 10-22; claim 26). In the preferred embodiment, the monoclonal antibodies are human monoclonal antibodies or produced by recombinant DNA methodology, for example, chimeric monoclonal antibodies (page 12, line 18, to page 15, line 24; examples; claims 27-29).

In one embodiment, the antibodies are administered when the individual presents with bloody diarrhea (page 8, line 25, to page 9, line 4). Alternatively, the antibodies are administered to the individual prior to the onset of symptoms (page 8, lines 16-24, such as bloody diarrhea, cerebral hemorrhage, seizures, or kidney damage. In other embodiments, the antibodies are administered when the individual is diagnosed with *E. coli* infection, or at the onset of hemolytic uremic syndrome (page 20, lines 5-16).

The claims in this appeal are specific to the dosage formulation per se, and are drawn to antibodies to specific subunits (page 7, lines 10-18; pages 16-60, especially pages 44-45; claims 30 and 33) and specific dosages (claims 34-36) which could be determined only by the use of an animal model that was actually predictive of human

infection (the neonatal pig) using bacterial strains obtained from humans with severe disease that at least in some cases was fatal (examples at pages 16-60).

It must be emphasized that the data in the application was essential for one of skill in the art to know that antibodies to a Stx2 IN HUMANS would be effective in treating or preventing HUS, that antibodies to a SINGLE SUBUNIT of Stx2 IN HUMANS would be effective in treating or preventing HUS, and what kind of actual DOSAGES would be useful.

The examiner's rejections are all premised on whether or not one skilled in the art could predict, with a reasonable expectation of success, that which is claimed, from prior art that provides no data whatsoever with respect to *E. coli* infection IN HUMANS. Due to the unique nature of this disease, which leads to HUS, once simply cannot do this. This is why multiple declarations were submitted in response to the rejections, showing that independent, unpaid third parties all of whom are above ordinary skill in the art, felt that one must have a model predictive of success in HUMANS, and actual data, to reach that which is claimed.

This technology is very important. It is currently being developed using non-profit research funds due to the critical need for such a product, a need which has been known for many years but for which there is still no accepted product available to clinicians. This is yet further evidence of the non-obviousness of this product.

(b) Rejection Under 35 U.S.C. § 103

Claims 26-36 were rejected under 35 U.S.C. § 103(a) as obvious over U.S. Patent No. 5,512,282 to Krivan *et al.* ("Krivan") and Perera *et al.* J. Clin. Microbiol. 26(10):2127-2131 (1988) ("Perera") in view of WO 90/07861 by Protein Design Labs,

Inc ("Queen") and Engelman *et al.* "Human Hybridomas and Monoclonal Antibodies ed. Engleman, Foung, Larrick, Raubitschek (Plenum Press 1985) pp. 95-112 ("Engelman") and further in view of U.S. Patent No. 6,080,400 to Williams.

None of the prior art teaches one skilled in the art to use an antibody to only a single subunit of the Stx2 for treatment or prevention of disease. None of the prior art recognizes that one only has to block Stx2 IN HUMANS to prevent the mortality and other extremely serious complications of HUS associated with certain highly virulent strains of *E. coli*. None of the prior art recognizes that the toxins associated with strains of *E. coli* that infect humans, as compared to other animals, are different, and that antibodies to toxin from animal strains may not be effective in treating or preventing complications of HUS. None of the prior art recognizes that one can block only a single subunit and still treat or prevent the high mortality or serious complications of HUS. Without recognizing these aspects, and identifying a useful animal model, one cannot predict an effective dosage, much less the dosage ranges defined by the dependent claims.

As discussed in more detail below, the art recognizes that antibodies should be useful in the treatment of HUS (Krivan, Williams). The art recognizes that one can make antibodies to just one subunit (Perera) which are useful in diagnostics. There is nothing that would lead one to substitute these subunit specific antibodies into Krivan, determine an effective dosage, and then have a reasonable expectation of success. None of the art demonstrates any successful treatment or prevention of clinical symptoms.

Therefore one skilled in the art would not be lead either to select antibodies as defined by all claims now pending, not just claim 30 as previously presented, nor to have a reasonable expectation of success if one did so.

i. The Legal Standard for Obviousness

"References relied upon to support a rejection under 35 USC 103 must provide an enabling disclosure, i.e., they must place the claimed invention in the possession of the public." *Application of Payne*, 606 F.2d 303, 314, 203 U.S.P.Q. 245 (C.C.P.A. 1979); *see Beckman Instruments, Inc. v. LKB Produkter AB*, 892 F.2d 1547, 13 U.S.P.Q.2d 1301 (Fed. Cir. 1989). A publication that is insufficient as a matter of law to constitute an enabling reference may still be relied upon, but only for what it discloses. *See Reading & Bates Constr. Co. v. Baker Energy Resources Corp.*, 748 F.2d 645, 651-652, 223 U.S.P.Q. 1168 (Fed. Cir. 1984); *Symbol Technologies, Inc. v. Opticon, Inc.*, 935 F.2d 1569 (Fed. Cir. 1991).

"Focusing on the obviousness of substitutions and differences, instead of on the invention as a whole, is a legally improper way to simplify the often difficult determination of obviousness." *Gillette Co. v. S.C. Johnson & Sons, Inc.*, 919 F.2d 720, 724, 16 U.S.P.Q.2d 1923 (Fed. Cir. 1990); *see Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1383, 231 U.S.P.Q. 81, 93 (Fed. Cir. 1986). "One cannot use hindsight reconstruction to pick and choose among isolated disclosures on the prior art to deprecate the claimed invention." *In re Fine*, 837 F.2d 1071, 1075 (Fed. Cir. 1988). The prior art must provide one of ordinary skill in the art with the motivation to make the proposed modifications needed to arrive at the claimed invention. *See In re Geiger* , 815 F.2d 686, 2 U.S.P.Q.2d 1276 (Fed. Cir. 1987); *In re Lalu and Foulletier*, 747 F.2d 703,

705, 223 U.S.P.Q. 1257, 1258 (Fed. Cir. 1984). Claims for an invention are not *prima facie* obvious if the primary references do not suggest all elements of the claimed invention and the prior art does not suggest the modifications that would bring the primary references into conformity with the application claims. *In re Fritch*, 23 U.S.P.Q.2d, 1780 (Fed. Cir. 1992). *In re Laskowski*, 871 F.2d 115 (Fed. Cir. 1989). This is not possible when the claimed invention achieves more than what any or all of the prior art references allegedly suggest, expressly or by reasonable implication. Here, the references do not teach one skilled in the art to select an antibody to a subunit of Stx2, in a therapeutically effective dosage, to treat or prevent HUS.

ii. The Claimed Invention

The claims define a formulation containing an effective dosage of antibodies reactive with a single subunit of Stx2, which neutralize Stx2, thereby preventing or treating hemolytic uremic syndrome ("HUS") in a human. The effective dosage for humans could be determined only using the neonatal pig model. See the example at pages 19-24. Absent this animal model, which is the only one predictive of human disease, one could not have determined an effective amount, the examiner's unsupported conclusions to the contrary. This is important to all of the claims on appeal, especially those defining a specific dosage, including claims 26 (effective amount), 31 (prevent neurological symptoms of HUS), 32 (prolong survival), and 34-36, which define actual dosages which were empirically determined to be effective.

The application demonstrates the efficacy of antibodies immunoreactive with a single subunit in preventing HUS associated mortality. Figures 2 and 3 demonstrate that applicants' humanized antibodies are neutralizing antibodies and their subunit specificity.

Figures 4 and 6 show the average survival time of mice exposed to shiga like toxin II ("Stx2"), without treatment, and after treatment with humanized antibodies to subunit A of Stx2 (these results correlate with those in Figure 2, for antibody 5C12, which neutralizes Stx2 and Stx2A, and 6G3, which neutralizes Stx2 and Stx2A, showing that the antibody to the Stx2A is most effective). Sheoran, et al., "Tsx2-specific human monoclonal antibodies protect mice against lethal infection with *E. coli* expressing Stx2 variants" *Infect. Immun.* 71(6):3125-3130 (June 2003) was submitted as further evidence that the monoclonal antibodies to the individual subunits are protective, but that their activities are different depending on the specificity. As the data in the application and the paper demonstrates, the anti-Stx2 A antibodies have broader protection than the anti-Stx2 B antibodies. This is particularly relevant to claims 30 and 33, which are drawn to antibodies to specific subunits. Claims 30 and 33 are specific to particular subunits of the Shiga like toxin II. One could only determine the usefulness of the isolated subunits by actually making and then testing the antibodies to the subunits.

The prior art fails to teach any guidance as to (1) the selection of antibodies to Stx2 only to treat or prevent HUS, (2) that antibodies to a single subunit of Stx2 can be effective in preventing or treating disease (not just in a diagnostic assay) (as required by all of the claims on appeal) and (3) what constitutes an effective dosage of these antibodies (as defined functionally by claims 31 and 32, and by actual dosages by claims 34-36). It would not have been obvious from studies using animals such as mice what an effective dosage would be, since mice are very resistant to infection, requiring many times more toxin to become sick, than humans. Only pigs have been proven to be a good model for humans (it is the only other animal species that naturally develops systemic

complications when infected with Shiga toxin-producing *E. coli*), and therefore a model that allows determination of an effective dosage. Cattle cannot be used as animal models for human disease, since cattle do not contain the receptors on their blood vessels for the bacteria which infect humans, and therefore are not susceptible to the systemic disease as humans and piglets do. Therefore only studies conducted in pigs or humans can be used to determine the critical components of the disease causing etiological agent, what compounds would be effective to treat these critical components, and what the effective dosage of these compounds would be.

Moreover, the organisms isolated from cattle are antigenically distinct from the organisms isolated from humans. Even the terms are different. For example, the Shiga-like toxin II from *E. coli* infecting cattle is more similar to the Shiga-like toxin I of the *E. coli* infecting humans.

iii. The Prior Art:

Krivan

Krivan does not place one of skill in the art with antibodies to Stx2 which would be effective to treat or prevent ***human uremic syndome***. Krivan describes animal antibodies. It is not clear to what toxin - it appears that it is only to the SLT forms that cause animal disease, not to the Stx2 form causing HUS.

Perera does not teach antibodies for therapeutic use and suggests that antibodies to subunits of Stx2 are not as effective as antibodies to Stx1. An important aspect of the studies conducted by Appellants was the use of a strain of bacteria which *infects and causes disease in humans*. Krivan does not recognize that the strains infect different hosts, and therefore that one cannot extrapolate from reagents in one species, cattle, for

use in another, humans, even though the organism, *E. coli*, are the same, since there are differences in the toxins, and in the host species, cattle and humans which are infected differently and have different diseases.

Krivan teaches polyclonal, monospecific *bovine* antibodies for the detection of a Shiga-like toxin or for treating hemolytic uremic syndrome. There is no disclosure or suggestion in this reference to obtain a human monoclonal antibody that will bind to, and specifically neutralize, Stx2 from the *E. coli* in humans. Krivan does say one could treat humans - he does not say that one must use human antibodies, or antibodies to *E. coli* which infects and causes disease in humans. Indeed, Krivan does not even recognize that the bacteria that infect cattle are unable to infect humans and cause HUS. Therefore, Krivan does not disclose nor enable treatment of humans to prevent HUS.

Krivan says his antibodies and invention ***are not, and cannot be, useful in humans.*** As the following excerpt from the patent makes clear, the animals to be treated to make antibodies ***do not possess receptors for the toxin (thereby excluding humans), and the resulting antibodies therefore would not be administerable to humans (it is well known one cannot administer bovine antibodies by injection to humans):***

"To achieve the objects and accordance with the purpose of the invention, as embodied and broadly described herein, the present invention provides an antitoxin to one or more SLTs. It comprises purified IgG that contains high titer, monospecific polyclonal antibodies to a Shiga-like toxin. (col. 6, lines 17-21)

The antibodies can be purified from the IgG. Therefore, the invention also provides high titer, monospecific, purified polyclonal antibodies to an SLT. Preferably, the antibodies comprise bovine IgG." (col. 6, lines 22-26)

"As used herein, the term "Shiga-like toxin (SLT)" refers to any cytotoxin similar in both structure and function to Shiga toxin. Known SLTs include SLT-I, STX2, and STX2I. They also include known variants of STX2, which are STX2v, STX2vh, and STX2vp. The term encompasses the presently unknown SLTs or variants thereof that may be discovered in the future, since their characterization as an SLT or variant thereof will be readily determinable by persons skilled in the art." (col. 7, line 65 to col. 8, line 6)

"The purified IgG of the invention is made by a novel modification of standard techniques for making polyclonal antibodies by inoculating an animal with an antigen and recovering immunoglobulins from a fluid, such as serum, that contains the immunoglobulins after the animal has had an immune response. The inventors surprisingly and unexpectedly discovered that they were able to inoculate a bovine animal with a purified, preferably active, SLT without significant ill effect to the animal." (page 8, lines 7-15)

" Without wishing to be bound by theory, the inventors hypothesized that the cell membranes of the cells of such an animal do not contain a receptor for SLTs or only contain low levels of receptors, when compared to other mammals or humans. Presumably, this allows high amounts of purified, active toxin to be inoculated into the animal and presumably allows the toxin to remain in unbound form longer in the animal, thereby creating a much greater antigenic response." (col. 8, lines 16-24)

"***Therefore, the method of the invention is applied to any animal that has few or no receptors to SLTs. Such animals can be identified by those skilled in the art through standard techniques involving the injection of an SLT into the animal and the***

observation of its effect on the animal and the titer of antibodies produced by the animal. " (emphasis added) (col. 8, lines 25-30)

Accordingly, there is no teaching in Kriven of the need to make human or humanized antibodies to STX2, no teaching of how to make such antibodies, no recognition that this is the critical toxin to protect against, much less what an effective dosage is (as defined by claim 26), and even less so that one might target a specific subunit of the toxin as required by all of the claims, and specifically either subunit A (claim 30) or subunit B (claim 33). Polyclonal antibodies would not be specific to a single subunit ***unless one actively took the step of removing antibodies from the mixture which are reactive with the other subunit and the combination of subunits.***

Krivan teaches away from treating humans by stating that the method is for the treatment of animals that have few or no receptors to SLTs. Humans have receptors. That is why cattle and humans are different.

Williams

It is not clear that Williams is available as prior art, even under 35 U.S.C. 102(e), since it issued on an application filed March 13, 1997, well after appellants' priority date of November 15, 1996. Williams is drawn to the use of *avian* antibodies elicited by immunization of birds with recombinant, preferably cross-linked toxin fragments. These can allegedly be from subunit A or B of the toxin, which can be from a pathogenic strain of *E. coli*, including an *E. coli* which infects humans (col. 17). It is clear there is no stated preference between stx1 and stx2 (col. 20, lines 38-45) although studies in mice showed protection from lethality *only* by using antibody to stx1, even though the antibody was a neutralizing antibody, (col. 66) which is the complete opposite to what

appellant claims (antibodies to Stx2). From the discussion at col. 24, line 42 to col. 25, line 4, the patentees believed one can only obtain neutralizing antibodies by immunization to both subunits A and B, arranged in a conformationally correct manner, again the opposite of what is claimed, i.e, antibodies to a single subunit. Of course, their studies prove that just because an antibody is a neutralizing antibody, does not mean it protects from the toxin. There is no teaching or even recognition that one cannot inject avian antibodies into humans - see col. 27, line 62 to col. 28, line 44. The only immunization studies performed were in chickens and mice (col. 62-64), neither appropriate animal models for treatment or prevention of HUS. The only administration of antibodies was from rabbits into mice (example 19). However, the study was not done as it would occur in a natural state (oral infection) but by premixing toxin and antibody, then injecting it intraperitoneally into the mice. Col. 66. Therefore the results would not be predictive of efficacy in humans.

Perera

Perera is relied upon for its teaching of toxin neutralization. Perera teaches five monoclonal antibodies which bind to the -subunit of STX2 and were able to neutralize the toxin as assayed using HeLa cells or Vero cells *in vitro* (for example, see Materials and Methods, page 2128, 2 nd column). As noted at page 2130, col. 2, the antibodies are useful in diagnostics of disease. There is no mention of therapy other than to note that the shiga like toxins may play a role in disease "although no direct proof for the involvement of SLTs in pathogenesis has yet been demonstrated." Perhaps more importantly, page 2131, col. 1, discusses the relative specificity and sensitivity of the antibodies, and notes that none of the antibodies reactive only with Stx2 could be used to

detect organisms; antibodies to Stx2 which were effective were only able to be used to detect organisms producing both Stx1 and Stx2. Accordingly, one would not be led by Perera to use these antibodies in therapy, nor one would have a reasonable expectation of success using just an antibody to Stx2, much less to a single subunit of Stx2. The diagnostic results here clearly teach away from the use of the anti-Stx2 antibodies.

Perera even in combination with Krivan or Williams does not teach that these monoclonal antibodies alone would be effective in treating or preventing HUS, nor in what amount. There is not only no teaching of a therapeutic use, there is nothing that would lead one to estimate a dosage. Moreover, based on example 19 of Williams, the mere fact that an antibody is a neutralizing antibody (i.e., able to complex with antigen and neutralize charge so that a precipitate is formed) does not mean it will be therapeutically effective.

Queen and Engelman

Queen and Engelman were cited merely to show that humanized and/or recombinant antibodies could be made.

Queen generalizes as to the advantages of humanized antibodies over non-human antibodies. It should be noted that the advantages described therein, are generally directed to combinations of humanized light and heavy chains with donor immunoglobulin CDRs. These combinations are produced using recombinant genetic and biochemical techniques. The techniques do not incorporate the use of an intact "immune system" to produce such humanized monoclonal antibodies.

iii. Appellants have submitted Expert Evidence which the examiner has ignored

Although appellants do not believe the examiner has established a *prima facie* case of obviousness, in view of the faulty analysis of the references, they have submitted expert evidence to rebut the examiner's rejection. The examiner keeps saying that because Krivan *claims* treatment of humans it enables and makes obvious treatment of humans, but this is contrary to the evidence submitted by appellants, which the examiner never rebuts with facts, only unsupported assertions. The declarations of experts in this field have been submitted that state that the foregoing analysis by appellants of the prior art is accurate and that Krivan neither discloses, nor makes obvious, such a formulation. The examiner has improperly ignored this evidence.

It has been well established that the examiner should consider all rebuttal arguments and evidence presented by applicants. Rebuttal evidence may include evidence of 'secondary considerations, such as 'commercial success, long felt but unsolved needs, [and] failure of others, evidence that the claimed invention yields unexpectedly improved properties or properties not present in the prior art, or evidence that the claimed invention was copied by others. It may also include evidence of the state of the art, the level of skill in the art, and the beliefs of those skilled in the art. For example, rebuttal evidence may include a showing that the prior art fails to disclose or render obvious a method for making the compound, which would preclude a conclusion of obviousness of the compound. The examiner should not evaluate rebuttal evidence for its 'knockdown' value against the *prima facie* case or summarily dismiss it as not compelling or insufficient. If the evidence is deemed insufficient to rebut the prima facie

case of obviousness, the examiner should specifically set forth the facts and reasoning that justify this conclusion. In re Piasecki, 745 F.2d 1468 (Fed. Cir. 1984)

The evidence submitted by appellants is drawn to two areas: establishing the level of skill in the art and what the prior art teaches one of skill in the art. ... "[T]he point [is] that the legal conclusion of obviousness cannot be reached without an appreciation of the level of ordinary skill in the art, Graham v. John Deere Co. ... and that 'level' must be determined by a consideration of all evidence made available to the trier of the issue which is related to the state of the particular technology at a given point in time. When all the evidence is evaluated, it may well turn out and often does, that the level of skill was not quite what it appeared to be when only a portion of the evidence, e.g. printed patents or publications, was considered ... ."Most of the objective evidence presented by patent applicants to the Patent Office to establish non-obviousness, or, perhaps more correct in a procedural sense, to rebut a *prima facie* case of obviousness, is technical in nature--a comparison of the prior art products with the claimed products, for example. In re Palmer, 451 F.2d 1100, 172 USPQ 126 (CCPA 1971) "[T]his court ... has found patentable subject matter even where the invention is apparently simple in nature or quite 'close', on the surface, to the prior art, but where the small difference has eluded those of ordinary skill in the art in search of the solution to a persistent problem or where that difference unexpectedly yields an improved product or known product in an unexpectedly advantageous manner. *Id.*

The Declaration of Dr. Florian Gunzer:

Dr. Gunzer is a microbiologist in Germany, working on the virulence mechanisms of Shiga toxin producing *E. coli.* He has published in peer reviewed international

journals and is an Infectious Disease consultant with the department of pediatrics at the Hanover Medical School in Germany. He has been working on a swine model for human enteric infections since 1994. This is the swine model that was crucial for applicants' discoveries leading to the claimed formulations. He has recently verified that this model develops the same disease symptoms in humans including renal thrombotic microangiopathy, and concludes that it is unique in its potential for evaluating prophylactic or therapeutic approaches for HUS, including those factors critical for determining what an effective dosage and subunit specificity would be in humans.

The Declaration of Dr. John M. Leong

Dr. John M. Leong is an Associate Professor of Molecular Genetics and Microbiology at the University of Massachusetts Medical School, a former Pew Scholar in the Biomedical Sciences and a former Established Investigator of the American Heart Association. Dr. Leong states that there are two critical features leading to life-threatening complications by Shiga toxin producing strains of *E. coli* O157:H7: (1) secretion of shiga-like toxin, which is essential for the systemic manifestations of STEC infection, and (2) generation attaching and effacing (AE) lesions on the intestinal epithelium, lesions that disrupt the cytoskeleton of epithelial cells. He then also states that the only animal model for infections with *E. coli* O157:H7 and other serotypes of STEC is the neonatal gnotobiotic piglet. This is critical for one to determine an effective dosage and subunit specificity.

Declaration of Dr. Saul Tzipori

Dr. Tzipori is an inventor of this application. As he previously explained at the interview with the examiner, Dr. Tzipori spent two decades developing the pig model, to

determine the virulence factors of the *E. coli* that leads to hemolytic uremic syndrome (HUS). There are two important factors that only piglets and humans have: toxin receptors on blood vessels to produce disease symptoms such as kidney failure and brain damage by the absorbed toxin. The second key factor is the ability of the bacteria to cause attaching effacing (AE) lesions (damage to the gut wall) in pigs and in humans. This facilitates the absorption of the toxin from the severely damaged gut into the blood stream. Therefore only the piglets can be used as an animal model for this human disease. Moreover, only if the model has receptors on their blood vessels, and gut damaged by bacteria to facilitate absorption of the toxin, can one determine an effective dosage of the therapeutic. It is also critical for determination of subunit specificity.

Krivan does not describe a method of preventing HUS in humans. Krivan describes only the administration of polyclonal antibodies produced in cattle. These antibodies cannot be used to treat humans, nor is there any way to predict if they would be in the slightest way predictive of what could be done in humans. Appellants claim a composition of an effective amount of an antibody that is specific for a single subunit of Stx2 that can be administered by injection to a human to treat or prevent symptoms of HUS. Krivan's bovine antibodies cannot be administered to humans by injection since they would elicit an immune response. If one did administer them orally, the dosage would be very different from the dosage required for efficacy when administered by injection.

Dr. Tzipori attaches letters from two additional experts, Dr. Harley Moon of the College of Veterinary Medicine, Iowa State University, and Dr. Phillip I. Tarr, Professor of Pediatrics and Microbiology, Washington University School of Medicine in St. Louis,

that further demonstrate why the piglets are the only useful animal model and why such a model is critical to determine and characterize what an effective reagent is for treatment or prevention of HUS.

iv. The Prior art is not enabling nor does it provide the required motivation

As noted above, "References relied upon to support a rejection under 35 USC 103 must provide an enabling disclosure, i.e., they must place the claimed invention in the possession of the public." *Application of Payne*, 606 F.2d 303, 314, 203 U.S.P.Q. 245 (C.C.P.A. 1979); *see Beckman Instruments, Inc. v. LKB Produkter AB*, 892 F.2d 1547, 13 U.S.P.Q.2d 1301 (Fed. Cir. 1989). A publication that is insufficient as a matter of law to constitute an enabling reference may still be relied upon, but only for what it discloses. *See Reading & Bates Constr. Co. v. Baker Energy Resources Corp.*, 748 F.2d 645, 651-652, 223 U.S.P.Q. 1168 (Fed. Cir. 1984); *Symbol Technologies, Inc. v. Opticon, Inc.*, 935 F.2d 1569 (Fed. Cir. 1991).

Neither Krivan (bovine polyclonal antibodies) nor Williams (avian recombinant antibodies) places one of skill in the art with antibodies to STX2 which would be effective to treat or prevent HUS, and certainly not antibodies to a specific subunit of Stx2. None of the prior art teaches that one can treat or prevent symptoms in humans using an antibody to a single subunit of a single toxin, Stx2. Even if one skilled in the art were motivated to combine the teachings of Krivan and Williams, which is not provided by either, one would not have that which is claimed, nor could one possibly have any reasonable expectation of success. This could only come from having actual data from a suitable model for extrapolation to humans. This is critical with respect to the claims that

are drawn to the specific subunits, claims 30 and 33, to the claims for alleviation of specific symptoms, claims 31 and 32, and to the claims to defined dosages, claims 34-36.

Krivan only provides animal antibodies, and it is not clear to what toxin - it appears that it is only to the SLT forms that cause animal disease, not to the STX2 form binding to human receptors and thereby causing HUS in humans. William is focused on antibodies produced by immunization with a fusion peptide, and there is no evidence it could protect a human from developing symptoms Indeed, as noted above, Williams teaches one must have antibody to both subunits in order to have an efficacious antibody to any form of the bacteria in any species.

"Focusing on the obviousness of substitutions and differences, instead of on the invention as a whole, is a legally improper way to simplify the often difficult determination of obviousness." *Gillette Co. v. S.C. Johnson & Sons, Inc.*, 919 F.2d 720, 724, 16 U.S.P.Q.2d 1923 (Fed. Cir. 1990); *see Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1383, 231 U.S.P.Q. 81, 93 (Fed. Cir. 1986). "One cannot use hindsight reconstruction to pick and choose among isolated disclosures on the prior art to deprecate the claimed invention." *In re Fine*, 837 F.2d 1071, 1075 (Fed. Cir. 1988).

Here, the examiner has taken the answer - that STX2 from certain enterohemorrhagic *E. coli* is critical to development of HUS in humans, and the data generated by Appellants with respect to the discovery that antibodies to specific subunits can be effective, to say that what is claimed is obvious. He ignores the necessity for actual data in a predictive animal model for one to be led to what is claimed with a reasonable expectation of success. As Appellants and all of the independent third party experts have testified, it was critical to have a useful animal model to make any

determinations: that antibodies to Stx2 alone could be used to treat or prevent symptoms of HUS; that antibodies to only a single subunit could be used to treat or prevent symptoms of HUS (and especially antibodies to subunit A, defined by claim 30, which was shown to be important in a number of strains in which antibodies to subunit B was not effective), and what would be effective dosages of such antibodies(defined by symptoms, claims 31 and 32, or amount, defined by claims 34-36). The examiner has worked backwards from Appellants' data to find references from which he has selected isolated phrases to support his rejection. This is clearly improper.

The prior art must provide one of ordinary skill in the art with the motivation to make the proposed modifications needed to arrive at the claimed invention. *See In re Geiger*, 815 F.2d 686, 2 U.S.P.Q.2d 1276 (Fed. Cir. 1987); *In re Lalu and Foulletier*, 747 F.2d 703, 705, 223 U.S.P.Q. 1257, 1258 (Fed. Cir. 1984). Claims for an invention are not *prima facie* obvious if the primary references do not suggest all elements of the claimed invention and the prior art does not suggest the modifications that would bring the primary references into conformity with the application claims. *In re Fritch*, 23 U.S.P.Q.2d, 1780 (Fed. Cir. 1992). *In re Laskowski*, 871 F.2d 115 (Fed. Cir. 1989). This is not possible when the claimed invention achieves more than what any or all of the prior art references allegedly suggest, expressly or by reasonable implication.

As is clear from the expert Declarations, those skilled in the art believe one must have used an appropriate animal model to reach the claimed invention. Krivan in fact teaches away from this by teaching one should use an animal that does not have the receptors necessary for binding of the bacteria which causes disease in humans. The prior art must lead one to believe that antibodies to a single subunit of Stx2 could be

effective. The prior art does not do this, in any combination. Williams teaches away from such a composition by stating that the antibody should be made using a chimeric antigen of both subunits coupled together in a conformationally correct manner. The other references do not make up for these deficiences. Therefore the claimed methods cannot be obvious.

Moreover, as is very clear from the data in the application, that which is claimed achieves more than what would have been predicted. One could never have predicted that antibodies to subunit A (claim 30) would have efficacy against more strains than antibodies to only subunit B (claim 33) or the intact toxin.

The current rejections are analogous to the rejection deemed improper by the Federal Circuit. *See In re Deuel*, 34 U.S.P.Q.2d 1210 (Fed. Cir. 1995). In *Deuel*, the Court reaffirmed that a rejection based on an "obvious to try" standard was improper. The Court specifically found that prior art that teaches a method for obtaining a general result, when the actual results are unknown, is insufficient to make obvious the actual results obtained upon which the claims are based.

One of ordinary skill in the art would readily appreciate neutralization of Stx2 *in vivo* is absolutely critical to prevent or treat hemolytic uremic syndrome in a human. The prior art never describes neutralization of the toxin that prevents death and other severe symptoms, as claimed. The prior art describes neutralizing antibodies only in the classic sense - antibodies which react with antigen to form an immunoprecipitate. The appellants have provided a detailed analysis of toxin induced neurological signs and bacterial lesions; and prevention and treatment of such signs and lesions in piglets. It is important to realize the advantages that are gained by using piglet model systems. Piglets

require much smaller toxin doses; piglets can be infected orally with the bacteria; mice are very resistant to infection, requiring many times more toxin to become sick, than humans; cattle do not absorb toxin at all like humans. In contrast, toxin produced in the gut of piglets is taken up systemically, ***just as in children***, to cause systemic complications because the piglets have receptors to which toxin binds. This information, provided only by Appellants, is critical to determining a dosage effective to treat or prevent the symptoms of claims 31 and 32 and to provide the dosage ranges of claims 34-36.

There is no teaching in the cited references, singly or in combination, of an effective dosage to prevent or treat hemolytic uremic syndrome in a human, especially of an antibody to Stx2, and even less so to a single subunit of Stx2. It would not have been obvious from studies using animals such as mice what an effective dosage would be, since mice are very resistant to infection, requiring many times more toxin to become sick, much less from studies with cattle. Studies based on preincubation and injection are worthless in predicting success, only failure. The bacterial strains and therefore the toxins, as well as the hosts and the diseases, are very different. One skilled in the art cannot predict from cattle, pig and avian diseases, to treatment or prevention of human infection. Accordingly, the claimed method cannot be obvious.

v. The examiner has failed to separately examine the dependent claims

The examiner has "lumped" all of the claims together in his rejections, and failed to separately examine the dependent claims. These can be divided into three groups, those defining the antibodies as recombinant or humanized or chimeric antibodies, those

relating to the formulation dosage, including claim 31 drawn to an effective amount to treat or prevent neurological symptoms, and those drawn to specific subunits, A or B (where A is shown to be significantly more effective than B), claims 30 and 33, as discussed above.

The prior art does not teach administration of humanized (claim 27), recombinant (claim 28) or chimeric humanized antibodies (claim 29). The examiner has used hindsight to say that it would be obvious to substitute humanized, recombinant or chimeric antibodies for the antibodies described by Krivan or Williams. This is not what one skilled in the art is led to by Krivan, however. Krivan teaches oral administration, not administration by injection, although he recognizes antibodies can be administered in other ways including injection. Krivan teaches the importance of polyclonal antibodies, not monoclonal antibodies. Krivan therefore fails to provide any teaching of why one would want to make a recombinant or humanized antibodies, much less how one should make such antibodies or use such antibodies. Williams describes polyclonal antibodies made to a recombinant antigen, not a recombinant or humanized antibody and says it would be useful to inject a human with avian antibody. Neither Krivan nor Williams indicate there is any need to do so. One cannot use hindsight to say that even though the prior art says it is desirable to use bovine or avian polyclonal antibodies for treatment, which can be administered enterally, one should replace them with humanized or human monoclonal antibodies to one particular toxin, Stx2, even less so to a single subunit of Stx2, which can be administered in defined dosages by injection to a human. This simply is found no where in the art.

The art also fails to suggest using an appropriate animal model that would lead one of skill in the art to determine an effective dosage. The art leads one instead to believe that mice and cattle are predictive of human treatment, and indeed Krivan teaches away from use of an animal with receptors as is essential in human infection. The expert opinions submitted by appellant make it very clear this not the case.

The dosage Krivan provides is for oral administration; not parenteral (col. 10, lines 54-55). This amount, 100 mg to 5 grams, greatly exceeds the amount that would be parenterally administered to a human child. There is no dosage given in Williams. None of the secondary references make up for the deficiencies of either Krivan or Williams.

One must look at the claims to see that very specific dosages are defined:

34. The dosage formulation of claim 26 equivalent to 4 ml serum from an animal immunized with Shiga-like toxin II/kg body weight.

35. The dosage formulation of claim 26 producing a serum level of anti-Shiga toxin II antibodies of at least 0.5 micrograms/ml.

36. The dosage formulation of claim 26 equivalent to a dosage of 3 mg human monoclonal antibody to Shiga-like toxin II administered to a newborn pig.

One must first start with the claim from which these claims depend - which requires the use of antibodies to treat humans, which are specific to a single subunit of Stx2. None of the art teaches anything with respect to whether or not the antibodies must react with one or both subunits of the toxin that is described. If one did decide to use an antibody to a single subunit, it is clear from the studies described in the application that one would have to determine experimentally which of the two subunits was more

important to prevention of disease or symptoms associated therewith. Then one would have to determine the effective dosage for these antibodies to the selected subunit.

The only way one skilled in the art could arrive at the claimed formulations is using hindsight based on appellant's studies; it is not derivable from the prior art. This is not something that could be predicted with any degree of certainty - it required experimental verification. Only appellants' data provides the information that could lead one to the claimed subject matter.

(8) SUMMARY AND CONCLUSION

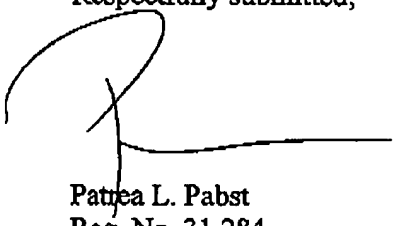
Krivan provides is a general teaching to treat or prevent disease in calves preferably administered orally, which is not suitable for injection into humans, and which is not specific to a particular subunit, nor in a dosage for administration by injection. Williams provides a recombinant peptide to immunize birds or rabbits. Williams teaches away from antibody to a single subunit by stating that it is very important to make antibody to an antigen consisting of two subunits coupled together to present the subunits in the same conformation as found in the native toxin (ie a conformation antigen).

The art does teach, nor lead one to, with a reasonable expectation of success, a formulation of antibodies to a single subunit of the Stx2 that causes HUS in humans, in a dosage that is effective to treat or prevent symptoms associated with debilitating disease or death. As the expert declarations demonstrate, those in the field did not know that one needed to target the shiga-like toxin II of the *E. coli* that infects humans, to prevent disease leading to death or kidney failure. This could not have been predicted based on data showing feeding of an immunoglobulin mixture to calves or pre-mixing toxin with

antibodies and injecting it intraperitoneally into mice. Neither calves nor mice are appropriate animal models for disease in humans since the disease proceeds differently due to the lack of receptors in the rumen of the calves, and the toxins are immunologically different. The only way one could have been led to the claimed subject matter is through extensive, careful, controlled studies conducted in a suitable animal model, as Appellants have done.

Based on the foregoing, claims 26-36 are not obvious in view of the cited art.

Respectfully submitted,



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APPENDIX OF CLAIMS ON APPEAL

26. (previously presented) A dosage formulation comprising an effective amount of human or humanized monoclonal antibodies, the antibodies consisting of antibodies neutralizing Shiga like toxin II *in vivo*, wherein the antibodies are specifically reactive with a single subunit of the Shiga like toxin II produced by *Escherichia coli* which causes hemolytic uremic syndrome, to prevent or treat hemolytic uremic syndrome in a human.

27. (previously presented) The dosage formulation of claim 26, wherein the antibodies are human monoclonal antibodies.

28. (previously presented) The dosage formulation of claim 26, wherein the antibodies are produced by recombinant DNA methodology.

29. (previously presented) The dosage formulation of claim 26, wherein the antibodies are chimeric monoclonal antibodies.

30. (previously presented) The dosage formulation of claim 26, wherein the antibodies bind to the alpha subunit of the Shiga like toxin II.

31. (previously presented) The dosage formulation of claim 26 wherein the antibodies are effective to prevent neurological signs of hemolytic uremic syndrome or lesions, wherein the neurological signs or lesions are selected from the group consisting of bloody diarrhea, acute renal failure, cerebral hemorrhaging, bacterial shedding into feces, bacterial lesions, paddling, head-pressing, ataxia, convulsions and wasting.

32. (previously presented) The dosage formulation of claim 26, wherein the antibodies are effective to prolong survival.

33. (previously presented) The dosage formulation of claim 26, wherein the antibodies bind to the beta subunit of the Shiga like toxin II.

34. (previously presented) The dosage formulation of claim 26 equivalent to 4 ml serum from an animal immunized with Shiga-like toxin II/kg body weight.

35. (previously presented) The dosage formulation of claim 26 producing a serum level of anti-Shiga toxin II antibodies of at least 0.5 micrograms/ml.

36. (previously presented) The dosage formulation of claim 26 equivalent to a dosage of 3 mg human monoclonal antibody to Shiga-like toxin II administered to a newborn pig.

APPENDIX OF EVIDENCE

(1) Sheoran, et al., "Tsx2-specific human monoclonal antibodies protect mice against lethal infection with *E. coli* expressing Stx2 variants" *Infect. Immun.* 71(6):3125-3130 (June 2003).

(2) The Declaration of Dr. Florian Gunzer

(3) The Declaration of Dr. John M. Leong

(4) The Declaration of Dr. Saul Tzipori with two letters from Dr. Harley Moom and Dr. Phillip I. Tarr

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Stx2-Specific Human Monoclonal Antibodies Protect Mice against Lethal Infection with *Escherichia coli* Expressing Stx2 Variants

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Shiga toxin-producing *Escherichia coli* (STEC) strains are responsible for causing hemolytic-uremic syndrome (HUS), and systemic administration of Shiga toxin (Stx)-specific human monoclonal antibodies (HuMAbs) is considered a promising approach for prevention or treatment of the disease in children. The goal of the present study was to investigate the ability of Stx2-specific HuMAbs to protect against infections with STEC strains that produce Stx2 variants. Dose-response studies on five HuMAbs, using the mouse toxicity model, revealed that only the three directed against the A subunit were protective against Stx2 variants, and 5C12 was the most effective among the three tested. Two HuMAbs directed against the B subunit, while highly effective against Stx2, were ineffective against Stx2 variants. In a streptomycin-treated mouse model, parenteral administration of 5C12 significantly protected mice up to 48 h after oral bacterial challenge. We conclude that 5C12, reactive against the Stx2 A subunit, is an excellent candidate for immunotherapy against HUS and that antibodies directed against the A subunit of Stx2 have broad-spectrum activity that includes Stx2 variants, compared with those directed against the B subunit.

Infection with Shiga toxin-producing *Escherichia coli* (STEC) strains is associated with hemolytic-uremic syndrome (HUS) (2, 24, 27), the leading cause of acute renal failure in young children (11). Shiga toxins (Stx) are cytotoxins and are major virulence factors of STEC. There are two immunologically distinct Stx types, known as Stx1 and Stx2, of which Stx1 is largely homogeneous, whereas the Stx2 group is highly heterogeneous and consists of at least 10 Stx2 gene variants (10, 14, 23, 31, 32, 37, 41, 42, 50). Stx2 is the most prevalent genotype identified in STEC isolated from patients with HUS (9, 40), and Stx2c is the most common Stx2 variant associated with HUS (9). Stx2 variants other than Stx2c are found frequently in asymptomatic STEC carriers but can often cause uncomplicated diarrhea (9) and rarely cause HUS (14, 33, 38, 45). Stx2f, identified in *E. coli* from pigeons, has been identified only once in humans, in a patient with diarrhea in Canada (10). In addition to pathogenicity to humans, Stx2 is more toxic to mice and piglets than Stx1. Stx2 is about 400 times more lethal to mice than Stx1 when administered systemically (44). STEC strains producing Stx2 alone cause more-severe neurologic symptoms in gnotobiotic piglets than STEC strains producing both Stx1 and Stx2, or Stx1 alone (8).

The nomenclature of Stx2 is confusing; Stx2vha and Stx2vhb (18), which are closely related to Stx2c (42), were originally identified as vtx2ha and vtx2hb (14). They were later shown to be activated by intestinal mucus (21) and named Stx2d (22). However, the Stx2d we refer to in the present study is the Stx2d cluster defined by Pierard et al. (37), which comprises Stx2d-OX3a (32), Stx2d-Ount (37), and Stx2d-O111 (33). An Stx molecule consists of a monomeric A subunit and a pentameric B subunit. Among STEC strains with different Stx2 variants, genetic differences in either the A or the B subunit or in both often confer antigenic and functional differences. The amino acid sequence identities of the A subunits of variants Stx2c (42), Stx2vha (14), Stx2vhb (14), Stx2d-OX3a (32), Stx2d-Ount (37), Stx2d-O111 (33), Stx2c (39), and Stx2f (47) with the A subunit of Stx2 are 100, 99, 99, 95, 93, 95, 94, and 71%, respectively. For the B subunit the amino acid sequence homologies are 96, 96, 96, 87, 88, 88, 87, and 82%, respectively.

The two current therapeutic approaches for HUS involve neutralization of Stx either in the gut or in the bloodstream. The two approaches attempted for Stx inactivation in the gut are (i) utilization of glycoconjugate polymers carrying Pk-trisaccharide sequences that serve as a receptor of Stx (1, 4, 5, 17) and (ii) use of recombinant bacteria displaying a Stx-specific glycolipid (globotriose or globotetraose) receptor (29, 30). We believe that systemic administration of Stx-specific neutralizing antibodies is currently the most promising approach for prevention or treatment of Stx-mediated systemic complications, including HUS (7) and edema disease in pigs (15). Murine Stx1- and Stx2-specific monoclonal antibodies (MAbs) have been shown to neutralize both toxins in vitro and in vivo (13, 28, 43). However, murine MAbs are not considered appropriate for human use. Reshaping of a murine antibody against Stx2 into a humanized form has recently been shown to completely protect mice against a lethal challenge with STEC when the antibody is administered within 24 h after infection (51). The disadvantage of a humanized antibody is that it still has mouse components and reduced affinity (12).

Mukherjee et al. have recently generated a panel of 50 human MAbs (HuMAbs) against Stx1 and Stx2 in transgenic

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mice (25, 26), from which we have selected a panel of 5 Stx2-specific HuMAbs that were shown to be highly protective for piglets, even when administered 12 h after an oral challenge with Stx2-producing STEC. In the present study, we used the mouse toxicity model (13, 25, 26, 28, 43) and the streptomycin-treated mouse model of STEC infection (22, 48, 49) to investigate the abilities of these five HuMAbs to protect against Stx2 variants.

MATERIALS AND METHODS

Bacteria. Enterohemorrhagic *E. coli* (EHEC) O91:H21 strain B2F1, which produces Stx2 variants Stx2vha and Stx2vhb (14), was obtained from the American Type Culture Collection (ATCC 51435). A streptomycin-resistant clone of wild-type B2F1 was produced by serially passaging B2F1 on a Luria-Bertani (LB) broth agar plate containing 30 to 100 μg of streptomycin/ml. EHEC O157 strains E32511 (producing both Stx2c and Stx2) (42) and 93-8059 (producing Stx2 only) were obtained from Andrew MacKenzie (Child and Youth Clinical Trial Network, Ottawa, Canada).

Crude preparation of Stx. A culture supernatant of B2F1 was used as a source of Stx2vha plus Stx2vhb (Stx2vha + Stx2vhb). A colony of wild-type B2F1 grown in 3 ml of LB broth for 7 h in a shaker at 37°C was transferred to a sterile flask containing LB broth at a dilution of 1/500 and incubated overnight in a shaker at 37°C. The culture was centrifuged at 1,750 × *g* for 30 min, and the supernatant was filter sterilized by passage through a 0.22-μm-pore-size filter. Similarly, a culture supernatant of E32511 was used as a source of Stx2c and Stx2, and a culture supernatant of 93-8059 was used as a source of Stx2.

Stx2-specific HuMAbs. Production of 37 hybridomas secreting Stx2-specific HuMAbs has been described elsewhere (25). Three HuMAbs against the A subunit (3E9, 2F10, and 5C12) and two against both the A and B subunits (5H8 and 6G3) have been shown to be the most efficient at neutralizing Stx2 in vitro and in vivo (25). These were selected for the present study. All five HuMAbs were of the human immunoglobulin G1(κ) [IgG1(κ)] isotype. HuMAb-containing ascites fluid was prepared by injecting hybridoma cells into the peritoneal cavities of pristane (Sigma-Aldrich Co.)-primed ICR SCID mice (Taconic, Germantown, N.Y.).

Quantitation of Stx2-specific HuMAbs by ELISA. The human IgG1(κ) concentration of each HuMAb in mouse ascites was measured by enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well plates were coated overnight at 4°C with 100 μl of the mouse MAb JDC-1 (IgG1 isotype) against human IgG1 (BD PharMingen) at 5 μg/ml. Plates were washed with phosphate-buffered saline–0.05% Tween 20 (PBS-T) and blocked with 100 μl of 2% nonfat dry milk powder in PBS-T/well at 37°C. After a wash, ascites samples diluted 1:100 in PBS-T were serially diluted twofold in duplicate rows of the plate (100 μl/well). A human IgG1(κ) (Sigma, St. Louis, Mo.) standard was similarly titrated on each plate from a starting concentration of 1 μg/ml. The plates were incubated at 37°C for 1 h and washed again. Horseradish peroxidase-conjugated goat anti-human IgG (Southern Biotech, Birmingham, Ala.), which was affinity purified and cross-adsorbed with human IgA, IgM, and IgD, was added at 100 μl/well at a dilution of 1/1,000. After incubation at 37°C for 1 h and a wash, plates were developed with a substrate solution (0.2% *o*-phenylenediamine–0.05% hydrogen peroxide in citric acid-phosphate buffer [pH 5.0]). The chromogenic reaction was stopped by using 50 μl of 2 M sulfuric acid, and absorbance was read at 490 nm. By using the linear portion of the IgG1(κ) standard curve, the total IgG1(κ) content of each HuMAb in ascites was determined and expressed as milligrams or micrograms of IgG1(κ) per milliliter of ascites fluid.

HeLa cell cytotoxicity neutralization assay. An in vitro HeLa cell cytotoxicity assay was used to evaluate the ability of each HuMAb to neutralize the toxic effects of Stx2vha + Stx2vhb exerted against HeLa cells. Briefly, HeLa cells were plated at 1.4×10^4/well on 96-well plates in McCoy's 5A medium (Mediatech, Inc., Herndon, Va.) containing 10% fetal bovine serum (Harlan Bioproducts for Science, Inc., Madison, Wis.) and incubated overnight at 37°C under 5% CO_2. A culture supernatant of B2F1 containing Stx2vha + Stx2vhb was titrated on HeLa cells to determine a dilution that killed ~70% of HeLa cells. Dead cells were removed by a wash with PBS, and crystal violet was used to stain viable cells (16). A mixture of the culture supernatant at a dilution that killed ~70% of HeLa cells and the HuMAb (5 μg/ml) or IgG1(κ) (5 μg/ml) as an isotype control (Sigma) was preincubated for 1 h at 37°C under 5% CO_2, then added to the cells, and incubated overnight at 37°C under 5% CO_2. A rabbit anti-Stx2 serum at a dilution of 1/400 was used as a positive control. The assay was similarly performed with a culture supernatant of EHEC O157 strain 93-8059 (a Stx2 producer), which served as another control. Plates were developed by crystal violet staining, and absorbance (optical density) was read at 690 nm. The percent neutralization of Stx2vha-, Stx2vhb-, and Stx2-mediated HeLa cell cytotoxicity by the HuMAb was then determined. Similarly, the HeLa cell cytotoxicity neutralization assay was performed utilizing culture supernatants of E32511 and 93-8059.

Mouse toxicity model. The mouse toxicity model (13, 25, 26, 28, 43) was used to determine the most efficacious HuMAb for neutralizing the effects of Stx2vha + Stx2vhb in vivo. Dose-response studies were performed with groups of 10 3- to 4-week-old female Swiss Webster mice (Taconic) to determine the amount of Stx2vha + Stx2vhb in the B2F1 culture supernatant required to induce 100% mortality in untreated animals. A volume of 160 μl of the B2F1 culture supernatant was sufficient (data not shown). The efficacies of HuMAbs were evaluated by administering every Stx2-specific HuMAb intraperitoneally (i.p.) to each of 10 3- to 4-week-old Swiss Webster mice at a dose of 1.25, 2.5, 5, 10, or 20 μg/mouse in 200 μl of PBS, followed 18 h later by i.p. administration of 160 μl of the B2F1 culture supernatant. A control group of 10 mice received human myeloma IgG1(κ) (20 μg/mouse; Sigma), and another control group received 200 μl of PBS alone. Both control groups were also challenged with 160 μl of the B2F1 culture supernatant. Mice were observed twice daily for survival.

Streptomycin-treated mouse model of STEC infection. A streptomycin-treated mouse model of STEC infection (22, 48, 49) was used to investigate the time-dependent efficacy of the most efficacious Stx2-specific HuMAb following infection with B2F1. Four-week-old DBA/2J mice were given drinking water containing 5 mg of streptomycin/ml for 24 h and were then denied food for 12 to 18 h. The mice received 10^{10} CFU of a streptomycin-resistant clone of B2F1 (0.1 ml) in 20% sucrose solution by oral administration. The animals were then permitted access to food and water containing 5 mg of streptomycin/ml ad libitum for the duration of the experiment (12 days). The efficacy of the most efficacious Stx2-specific HuMAb, 5C12, was tested following i.p. administration at a dose rate of 2.1 mg/kg of body weight following 0, 12, 24, 48, and 72 h of oral infection with 10^{10} CFU of B2F1. A group of 10 mice was used for each time point. A control group of 10 mice received human myeloma IgG1(κ) (30 μg/mouse injected i.p.; Sigma) at 0 h following infection with B2F1. Mice were observed three times per day for survival.

Immunoblotting. In addition to in vitro and in vivo neutralization of Stx2vha + Stx2vhb by HuMAbs, the reactivity of each HuMAb with Stx2vha + Stx2vhb was determined by immunoblotting. Stx2, purified as described elsewhere (6), and a 55-fold-concentrated culture supernatant of B2F1 as a source of Stx2vha + Stx2vhb were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions and electrophoretically transferred to a 0.2-μm-pore-size nitrocellulose membrane (Bio-Rad Laboratories, Richmond, Calif.). After transfer, the membrane was blocked with 5% nonfat dry milk powder in PBS-T at room temperature for 1 h, washed, and incubated with each HuMAb (2.5 μg/ml of PBS-T) at room temperature for 1 h. Human IgG1(κ) (Sigma) was used as a control. After a wash, strips were incubated with horseradish peroxidase-conjugated goat anti-human IgG (Southern Biotech) at a dilution of 1/1,000 for 1 h at room temperature and then washed and developed with the 3,3',5,5'-tetramethylbenzidine (TMB) peroxidase-substrate system (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.).

Statistical analysis. The grouped survival data were analyzed by the Mantel-Cox test and by using the PROC Freq procedure of SAS statistical software. Resulting *P* values of <0.05 were considered significant.

RESULTS

Reactivity in immunoblotting. HuMAbs 5C12, 3E9, and 2F10 reacted with the A subunits of Stx2 and Stx2vha + Stx2vhb (Fig. 1). HuMAbs 6G3 and 5H8 reacted strongly with the B subunit and mildly with the A subunit of Stx2 but did not react with any of the subunits of Stx2vha + Stx2vhb.

Neutralization of Stx2vha- and Stx2vhb-mediated HeLa cell cytotoxicity. Each of the Stx2-specific HuMAbs was effective at neutralizing the activity of Stx2 present in the culture supernatant of 93-8059; however, differences in relative potency were observed (5H8 and 6G3 showed the highest potency, followed, in descending order, by 5C12, 2F10, and 3E9) (Fig. 2). Similarly, consistent relative differences were observed among the Stx2 A-subunit-specific HuMAbs with respect to

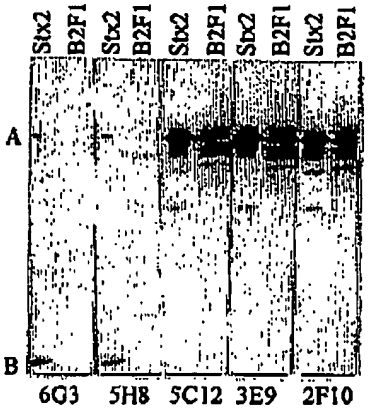

FIG. 1. Immunoblot reactivities of HuMAbs with Stx2 and Stx2 variants. Lanes Stx2 and lane B2F1 represent purified Stx2 and a concentrated culture supernatant of B2F1 containing Stx2 variants (Stx2vha and Stx2vhb), respectively. HuMAbs used to react with the Stx are given below the blot. Bands A and B represent the A and B subunits, respectively.

neutralization of Stx2vha + Stx2vhb present in the culture supernatant of B2F1 and neutralization of Stx2c present in the culture supernatant of E32511 (5C12 and 2F10 showed approximately equal potencies, while that of 3E9 was lower). In contrast, the B-subunit-specific HuMAb 5H8 did not neutralize Stx2vha + Stx2vhb or Stx2c, and 6G3 neutralized them at very low levels. Although 5C12 neutralized Stx2 completely, residual cytotoxicity was observed with Stx2vha + Stx2vhb and Stx2c; this might have been due to the presence of toxic factors other than Stx2 variants in the culture supernatants of B2F1

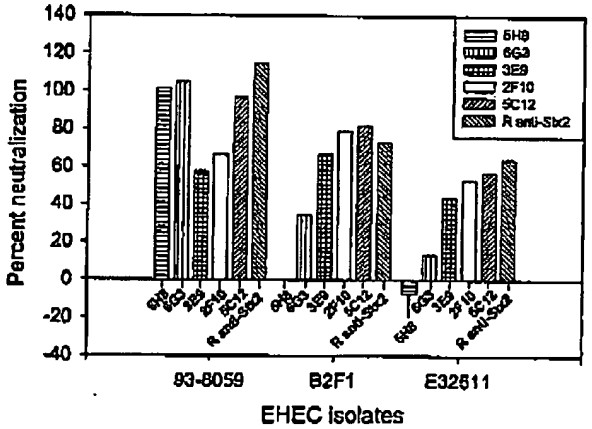

FIG. 2. Neutralization of HeLa cell cytotoxicity mediated by Stx2 (produced by 93-8059), Stx2vha + Stx2vhb (produced by B2F1), and Stx2 plus Stx2c (produced by E32511) by Stx2-specific HuMAbs. The B-subunit-specific HuMAbs 5H8 and 6G3 neutralized Stx2 completely. However, 5H8 did not neutralize Stx2vha, Stx2vhb, or Stx2c, and 6G3 neutralized them mildly. The A-subunit-specific HuMAb 5C12 and rabbit anti-Stx2 serum (R anti-Stx2) strongly neutralized all Stx types. The other A-subunit-specific HuMAbs, 2F10 and 3E9, were also very effective.

and E32511, since the rabbit anti-Stx2 serum also neutralized Stx2 completely but did not completely neutralize the Stx2 variants.

Neutralization of Stx2vha + Stx2vhb in vivo. Considering the identical in vitro neutralization patterns of the Stx2-specific HuMAbs against Stx2c and Stx2vha + Stx2vhb (Fig. 2), further studies to examine the relative potency of each HuMAb in vivo were performed only against Stx2vha + Stx2vhb, by utilizing the mouse toxicity model. At each dose, Stx2 A-subunit-specific HuMAbs 5C12, 2F10, and 3E9 significantly protected mice, as evidenced by comparison with the PBS control (average survival, 2.30 ± 0.35 days) and the HuMAb IgG1(κ) control (average survival, 2.35 ± 0.34 days) ($P < 0.0001$) (Fig. 3). In contrast, Stx2 B-subunit-specific HuMAbs 5H8 and 6G3 did not protect mice significantly at any dose level. HuMAbs 2F10 and 3E9 exhibited very similar dose-dependent effects on relative average survival; they did not differ significantly from each other at any dose level except 10 μg/mouse ($P < 0.0001$). In contrast, 5C12 did not show dose dependency; it protected 90% of the mice even at the lowest dose administered (1.25 μg/mouse). 5C12 provided better protection than 2F10 and 3E9, differing significantly from them at all dose levels except for 3E9 at doses of 10 and 2.5 μg/mouse. At the lowest dose (1.25 μg/mouse) tested, 5C12 was far superior to 2F10 and 3E9 ($P < 0.0001$).

Time-dependent efficacy of 5C12 in B2F1-infected mice. To test for the time-dependent efficacy of 5C12, mice were orally infected with 10^{10} CFU of B2F1, and HuMAb 5C12 was administered at 0 to 72 h after infection (Fig. 4). All control mice infected and treated i.p. at the same time with control human IgG1(κ) died, with an average survival time of 6 days. In contrast, 5C12 administered 0, 12, 24, or 48 h following infection protected 80% ($P = 0.0001$), 70% ($P = 0.0002$), 90% ($P < 0.0001$), or 60% ($P = 0.001$) of the mice, respectively (Fig. 4). However, 5C12 administered 72 h following infection protected only 20% of the mice, which was not a significant effect.

DISCUSSION

The main goals of the present study were (i) to identify the most effective Stx2-specific HuMAb by using the mouse toxicity model and (ii) to determine the protective ability of the selected HuMAb against Stx2c, the most prevalent Stx2 variant associated with HUS (9), by using the streptomycin-treated mouse model of oral STEC infection (22, 48, 49). Like others (18), we were unsuccessful in adapting the streptomycin-treated mouse model for strain E32511, which produces both Stx2 and Stx2c (data not shown). Consequently, we have used strain B2F1, described by other investigators (22, 48, 49, 51), which expresses both Stx2vha and Stx2vhb (14). B2F1 was ideal because the B subunits of Stx2c, Stx2vha, and Stx2vhb are identical and differ from the B subunit of Stx2 by 2 amino acids (14, 42). In addition, unlike the A subunit of Stx2c, which is identical to that of Stx2, the A subunits of Stx2vha and Stx2vhb differ by 3 amino acids from that of Stx2. Therefore, testing Stx2vha and Stx2vhb against our Stx2 A- and B-subunit-specific HuMAbs determined not only the influence of amino acid differences in the B subunit, but also that for the A subunit, on the relative protective abilities of the HuMAbs. Moreover,

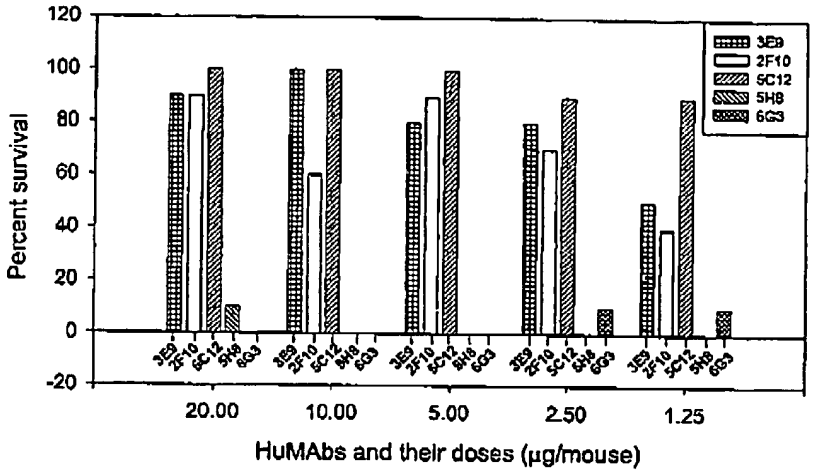

FIG. 3. Percent survival of mice given 20, 10, 5, 2.5, or 1.25 µg of HuMAb 3E9, 2F10, 5C12, 5H8, or 6G3 i.p., followed 18 h later with a 100% lethal dose of the Stx2vha + Stx2vhb-containing culture supernatant of EHEC isolate B2F1, also given i.p. Stx2 A-subunit-specific HuMAbs 5C12, 2F10, and 3E9 significantly protected mice relative to the PBS control (average survival, 2.30 ± 0.35 days) and the HuMAb IgG1(κ) control (average survival, 2.35 ± 0.34 days) ($P < 0.0001$). HuMAbs 5H8 and 6G3 did not protect mice. HuMAbs 2F10 and 3E9 exhibited very similar dose-dependent effects on relative average survival; they did not differ significantly from each other at any dose level except 10 µg/mouse ($P < 0.0001$). In contrast, 5C12 did not show dose dependency; it protected 90% of the mice even at the lowest dose administered (1.25 µg/mouse). At the lowest dose (1.25 µg/mouse) tested, 5C12 was far superior to 2F10 and 3E9 ($P < 0.0001$).

STEC strains producing Stx2vha + Stx2vhb have also been associated with HUS (14).

We first evaluated the neutralizing abilities of the five selected HuMAbs against Stx2 variants (Stx2c and Stx2vha + Stx2vhb) in vitro by HeLa cell cytotoxicity neutralization assay, followed by dose-response studies in the mouse toxicity model (13, 25, 26, 28, 43). Of the five HuMAbs tested (three A-subunit and two B-subunit specific), 5C12 was the most effective, and therefore it was selected for further evaluation in the streptomycin-treated mouse model of infection (22, 48, 49).

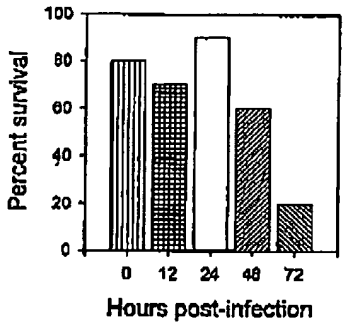

FIG. 4. Percent survival of mice orally infected with Stx2vha + Stx2vhb-producing B2F1 and given 5C12 i.p. at a dose of 2.1 mg/kg of body weight at various times postinfection. All control mice infected and treated i.p. at the same time with control human IgG1(κ) (2.1 mg/kg of body weight) died, with an average survival time of 6 days. In contrast, 5C12 administered 0, 12, 24, or 48 h following infection protected 80% ($P = 0.0001$), 70% ($P = 0.0002$), 90% ($P < 0.0001$), or 60% ($P = 0.001$) of the mice, respectively. However, 5C12 administered 72 h following infection protected only 20% of the mice, which was not significant.

5C12 was administered at various time points after bacterial challenge, since treatment of patients with STEC infection is expected to occur after exposure to infection, at the onset of bloody diarrhea. Studies with piglets have already shown that these HuMAbs are protective even when given after an oral bacterial challenge with Stx2-producing STEC (25). This is the first report, however, which shows that administration of a specific HuMAb against Stx2 (5C12 at 2.1 mg/kg) can significantly protect mice when given as long as 48 h after bacterial challenge. In contrast, a study using the same mouse model and strain B2F1 has shown that the Stx2-specific humanized MAb TMA-15, given at a dose of 1.0 mg/kg, protects mice when given as long as 24 h after bacterial challenge (51). It is possible that the differences in length of protection afforded by 5C12 and TMA-15 are due to differences in their respective affinities. Although concentration of Stx2 variants in the blood were not determined in the present study, Yamagami et al. (51) have reported that serum Stx2 variant levels are highest in mice at 48 h after STEC infection (51). This suggests that 5C12 can significantly protect mice even when the maximum levels of Stx2 variants are present in the bloodstream. The time window of 48 h for immunotherapeutic intervention has direct implications for children at risk of developing HUS (e.g., those presenting with bloody diarrhea or excreting STEC) and for individuals in contact with them. The development of rapid and sensitive diagnostic methods has made it possible to detect STEC infections almost a week before symptoms of HUS become apparent (34, 35).

The three amino acid differences between the A subunits of Stx2vha and Stx2vhb, on the one hand, and the A subunit of Stx2, on the other (14), did not significantly affect the binding of any of the Stx2 A-subunit-specific HuMAbs; all of them neutralized Stx2vha + Stx2vhb both in vitro and in vivo. How-

ever, the Stx2 B-subunit-specific HuMAbs (5H8 and 6G3) failed to neutralize Stx2vha + Stx2vhb, suggesting that one or both of the amino acid changes in the B subunits of Stx2vha and Stx2vhb considerably affected the neutralizing capabilities of 5H8 and 6G3. Since the B subunits of Stx2vha and Stx2vhb are identical to the Stx2c B subunit, 5H8 and 6G3 also failed to neutralize Stx2c in vitro. However, Stx2 A-subunit-specific HuMAbs neutralized Stx2c in vitro, because Stx2 and Stx2c have identical A subunits. The failure of 6G3 and 5H8 to neutralize Stx2c in vitro and their stronger immunoblot-reactivity with the B subunit than with the A subunit of Stx2 unequivocally show that the neutralization activities of these two HuMAbs are due to their binding with the B subunit and not the A subunit.

Given that STEC can produce any combination of Stx1, Stx2, and/or Stx2c (9), an ideal therapeutic formulation should, in our view, include HuMAbs specific for Stx1, Stx2, and Stx2c. Mukherjee et al. have recently reported production of protective Stx1-specific HuMAbs (26) for inclusion in such a formulation. Since it appears from this study that A-subunit-specific Stx2 antibodies display inhibitory activity against Stx2c as well, the selection of 5C12 combined with an effective Stx1-specific HuMAb, described in an earlier study (25), could provide broad-spectrum protection against Stx1, Stx2, and Stx2c. However, the efficacy of 5C12 needs to be further investigated in the orally infected piglet model, since piglets are the only species in addition to humans that are naturally susceptible to the systemic effects of Stx produced by *E. coli* strains that proliferate in the gastrointestinal tract (19, 20), with characteristic attachment-and-effacement lesions (36, 46), which are absent in the mouse (18). The mouse model is also less susceptible to Stx, as judged by the amount of toxin required to cause death compared to that for the piglet (3, 26) and presumably for children. The mouse infection model, however, is useful for screening and evaluation, because it is genetically uniform, available in large numbers, easy to manipulate, requires smaller amounts of reagents, and is less expensive and less labor-intensive. The piglet model, on the other hand, is more appropriate for preclinical evaluation of formulations and for validation, including determination of the likely effective therapeutic dose for humans.

We conclude that 5C12, which is reactive against the Stx2 A subunit, is an excellent candidate for immunotherapy against HUS and that antibodies directed against the A subunit of Stx2, as opposed to those directed against the B subunit, have broad-spectrum activity that includes Stx2 variants.

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Editor: J. D. Clements

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Saul Tzipori, Ramaswamy Balakrishnan and Arthur Donohue-Rolfe

Serial No.: 10/041,958 Art Unit: 1645

Filed: January 2, 2002 Examiner: Mark Navarro

For: *HUMAN NEUTRALIZING ANTIBODIES AGAINST HEMOLYTIC UREMIC SYNDROME*

Assistant Commissioner for Patents
Washington, D.C. 20231

DECLARATION UNDER 37 C.F.R. 1.132

Dear Sir:

I, Florian Gunzer, MD, hereby state:

1. I am a medical microbiologist at Hannover Medical School in Hannover, Germany. I currently work as a research scientist, focused on elucidating virulence mechanisms of Shiga toxin producing *Escherichia coli* (STEC) and enterohemorrhagic *Escherichia coli* (EHEC) causing human disease. I am using *in vitro* systems such as tissue culture and array analysis as well as animal models for *in vivo* investigation. I have published my work in several peer reviewed international scientific papers. I do also have an appointment as infectious disease consultant with the department of pediatrics at Hannover Medical School.

Serial No.: 10/041,958
Filed: January 2, 2002
DECLARATION UNDER 37 C.F.R. 1.132 OF DR. GUNZER

2. I have no interest in the above-identified patent application nor have I been compensated for my time.

3. I have worked since 1994 with the swine model for human infection with enteric pathogens. The neonatal gnotobiotic colostrum deprived piglet is uniquely relevant as a model to study human infections with O157 and non-O157 STEC/EHEC. In humans, oral infection with STEC/EHEC may cause severe enteritis with bloody diarrhea and, in certain circumstances, hemorrhagic colitis, followed in up to 10 % of cases by an extraintestinal complication, the hemolytic uremic syndrome (HUS). Hemolytic uremic syndrome is characterized through a triad of symptoms, anemia, thrombocytopenia, and acute renal failure due to vascular lesions, described as thrombotic microangiopathy (TMA). Thrombotic microangiopathy is the morphological hallmark of all forms of hemolytic uremic syndrome. STEC/EHEC produces several virulence factors among which a family of phage encoded cytotoxins, called Shiga toxin 1 or Shiga toxin 2, appears to be most important. Enteric manifestations of STEC/EHEC infection are attributed in part to the attaching and effacing (A/E) phenotype of the pathogens. Formation of A/E lesions requires expression of genes encoded by the LEE (locus of enterocyte effacement) region in the STEC/EHEC genome. The intimate enterocyte attachment of these organisms is thought to be critical for intestinal colonization and in facilitating transport of Shiga toxins from the intestine into the bloodstream where (in a proportion of patients) it

causes the systemic vascular renal and neurologic damage characteristic of HUS.

4. In a recent publication from my laboratory (F. Gunzer et al., Am.J.Clin.Pathol. 2002, 118:364-375) we could show for the first time, that neonatal gnotobiotic colostrum deprived piglets developed renal TMA, the hallmark of HUS in humans, following infection with either an O157:H7 or an O26:H11 EHEC strain. In addition to these vascular alterations, we observed A/E lesions in the gut and microhemorrhages in the CNS, pathologic changes that had been described by other investigators before. The clinical response of gnotobiotic piglets very closely resembled intestinal and extraintestinal features of human EHEC disease. After oral uptake of the pathogens, the natural route of infection, the animals developed diarrhea during a prodromal period, followed by transport of Shiga toxin to the bloodstream in sufficient quantities to cause systemic vascular damage, clinically apparent systemic manifestation of disease and death

5. For the above reasons neonatal gnotobiotic colostrum deprived piglets have a unique potential as a model to evaluate prophylactic or therapeutic approaches offering new advantages to prevent or lessen systemic complications of EHEC infection in humans.

6. I declare that all statements made herein of my own knowledge and belief are true and that all statements made on information and belief are believed to be true, and further, that the statements are made with the

Serial No.: 10/041,958
Filed: January 2, 2002
DECLARATION UNDER 37 C.F.R. 1.132 OF DR. GUNZER

knowledge that willful false statements are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Florian Gunzer, MD

Date: April 1, 2003

ATL1 #569711 v1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Saul Tzipori, Ramaswamy Balakrishnan and Arthur Donohue-Rolfe

Serial No.: 10/041,958 Art Unit: 1645

Filed: January 2, 2002 Examiner: Mark Navarro

For: *HUMAN NEUTRALIZING ANTIBODIES AGAINST HEMOLYTIC UREMIC SYNDROME*

Assistant Commissioner for Patents
Washington, D.C. 20231

DECLARATION UNDER 37 C.F.R. 1.132

Dear Sir:

I, John M. Leong, M.D., Ph.D., hereby state:

1. I am Associate Professor of Molecular Genetics and Microbiology at the University of Massachusetts Medical School. I am a former Pew Scholar in the Biomedical Sciences and a former Established Investigator of the American Heart Association. I have published numerous papers in peer reviewed journals, including an invited commentary in 2002 on a toxin produced by the enteric pathogen *Campylobacter jejuni* in the journal Science and a 2003 review on colonization by enterohemorrhagic *E. coli* O157:H7 in the journal Current Opinions in Microbiology.

2. I have no interest in the above-identified patent application nor have I been compensated for my time.

3. The life-threatening complications of human infection by Shiga Toxin Producing Strains of *E. coli* 0157:H7 (STEC) are hemorrhagic colitis and the triad of hemolytic anemia, thrombocytopenia, and kidney failure known as hemolytic uremic syndrome (HUS). In my expert opinion, the features that are central to the ability of STEC to cause this local and systemic damage are the ability to: (1) secrete shiga-like toxin, which contributes to intestinal damage and, through its toxicity to vascular endothelium, is essential for the systemic manifestations of STEC infection, and (2) generate attaching and effacing (AE) lesions on the intestinal epithelium, lesions that disrupt the cytoskeleton of epithelial cells. This disruption compromises intestinal epithelial integrity and is likely to promote the systemic absorption of shiga-like toxin produced by bacteria in the gut.

4. The neonatal gnotobiotic piglet is the only animal model for infections with *E. coli* O157:H7 and other serotypes of STEC that reproduces both of these critical elements of STEC pathogenesis. STEC O157:H7 generate AE lesions on intestinal epithelium, cause hemorrhagic colitis and systemic damage, mainly neurological via vascular damage in the central nervous system by shiga like toxin absorbed from the gut. (Some small animals, e.g. the mouse, are susceptible to systemic effects of shiga-like toxin, but do not manifest AE lesions upon intestinal infection—thus, some bacterial mutants

that are incapable of causing disease in humans are likely to be fully virulent in these animals.) As such, the gnotobiotic piglet is the best model for evaluating therapies for the prevention or treatment of tissue damage by STEC infection, in particular systemic manifestations due to endothelial damage by shiga-like toxin. Accurate estimation of the efficacious dose(s) of prophylactic or therapeutic agent to be administered to human patients, and the timing of those doses, is best determined in gnotobiotic piglets.

5. I declare that all statements made herein of my own knowledge and belief are true and that all statements made on information and belief are believed to be true, and further, that the statements are made with the knowledge that willful false statements are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

John M. Leong, M.D, Ph.D.

Date: 3/27/03

ATL1 #569711 v1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Saul Tzipori, Ramaswamy Balakrishnan and Arthur Donohue-Rolfe

Serial No.: 10/041,958 Art Unit: 1645

Filed: January 2, 2002 Examiner: Mark Navarro

For: *HUMAN NEUTRALIZING ANTIBODIES AGAINST HEMOLYTIC UREMIC SYNDROME*

Assistant Commissioner for Patents
Washington, D.C. 20231

DECLARATION UNDER 37 C.F.R. 1.132

Dear Sir:

I, Saul Tzipori, DVM, PhD, DSc, FRCVS, hereby state:

1. I am a Professor of Microbiology and Head of the Division of Infectious Diseases, at Tufts University School of Veterinary Medicine in Massachusetts.

3. I have conducted original scientific research on the prevention of systemic complications in *Escherichia coli* O157:H7 infection over the last two decades, using all the known laboratory techniques and the currently existing animal models including the mouse and the piglet models.

4. Diarrhea followed by systemic disease occurs only in humans and pigs when infected with the *E. coli* bacteria that produce Shiga toxin or Stx. The bacteria induce serious damage to the gut, which results in diarrhea in both humans and piglets. The Stx which is liberated by the bacteria in the gut is absorbed from the damaged gut in humans and piglets into the blood stream where it can damage blood vessels. In humans this

damage can lead to hemolytic uremic syndrome (HUS) manifested as kidney failure. In piglets, absorbed Stx causes diarrhea and brain damage. The only animal that has the receptors required for absorption of Stx is the piglet. Therefore piglets are the only model which can be used to determine the therapeutic dose against the systemic effect of the Stx. This includes the amount of human monoclonal antibody against Stx required to protect patients presenting with HUS, or diarrhea, or infected with, or exposed to the Stx-producing *Escherichia coli* bacteria. No other animal models including mice develop damaged gut and diarrhea after infection.

5. We consistently protect piglets experimentally infected with the bacteria well after they develop diarrhea, and before the onset of the brain injury and neurological symptoms. This mimics the situation in patients who can similarly be treated with the antibody after they present with diarrhea and before the onset of HUS which occurs 4-6 days later. We have determined that 5micrograms of Stx antibody must be present in each ml of blood to fully protect a single piglet from developing neurological symptoms and death. This requires a dose of 3mg of antibody per each kg of body weight. Based on these experiments, patients presenting with diarrhea, will similarly require to have 5 micrograms/ml of antibody circulating in their blood to be fully protected against the development of HUS. The exact injectable dose required to establish this amount of antibody in the blood stream of human individuals will be determined in a dose-response study during phase I clinical trials.

6. Given the incidence of HUS in the population, without studies in piglets it will take 10-12 years to determine the effective dose through Phase II/III clinical trials in humans. These bacteria do not cause gut damage or diarrhea in other animals including

the mouse model which is used by other investigators. The lack of gut damage and diarrhea reduce considerably the susceptibility of mice to Stx, and consequently alters the amount of antibody needed to protect them. The relative amount needed to protect a mouse will therefore be very different than that needed to protect a more susceptible host such as humans or piglets.

7. I have reviewed U.S. patent No. 5,512,282 to Krivan, et al. Krivan does not describe a method of preventing HUS in humans. Krivan et al describe the oral administration of polyclonal antibodies produced in cattle which are suitable for treating Stx-related diseases in animals. Unquestionably, polyclonal antibodies made in animals, however purified, cannot be injected into the blood stream of humans, either for treatment or prevention. More importantly, there is no evidence that specific antibodies, be it polyclonal or monoclonal, are effective at all when given orally, nor that they can prevent or protect against a systemic disease caused by toxin present in the blood stream. They will be digested and metabolized in the gut, even when the antibodies are administered either encapsulated, conjugated or emulsified. While they provide many examples to show how these polyclonal antibodies may be useful as diagnostic reagents for the detection of toxins in food products or stool, they provide no evidence what so ever as to how the administration of such antibody might safely and effectively protect, ameliorate, or prevent Stx-mediated systemic disease.

8. Attached are letters written by two experts in this field, Dr. Harley W. Moon of the College of Veterinary Medicine at Iowa State University, and Dr. Phillip I. Tarr, Professor of Pediatrics and Microbiology, Washington University School of Medicine in

Serial No.: 10/041,958
Filed: January 2, 2002
DECLARATION UNDER 37 C.F.R. 1.132

St. Louis, in support of the unique role of the pig model in testing agents and determining the effective dosages for the treatment or prevention of HUS.

9. I declare that all statements made herein of my own knowledge and belief are true and that all statements made on information and belief are believed to be true, and further, that the statements are made with the knowledge that willful false statements are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

S. Tzipori

Saul Tzipori, DVM, PhD, DSc, FRCVS

Professor of Microbiology

Date: 4/10/03

ATL1 #570336 v1

IOWA STATE UNIVERSITY
OF SCIENCE AND TECHNOLOGY

College of Veterinary Medicine
Department of Veterinary Pathology
Ames, Iowa 50011-1250
515 294-3282
FAX 515 294-5423

March 12, 2003

To Whom It May Concern:

Subject: Swine Model for Human Infection with Shiga Toxin Producing (STEC) Strains of *E. coli* O157:H7

The neonatal gnotobiotic or colostrum deprived piglet is uniquely relevant as a model for human infections with *E. coli* O157:H7 and other serotypes of STEC.
In humans, *E. coli* O157:H7 causes intestinal infection and diarrheal disease leading in some cases to hemorrhagic colitis. These enteric manifestations of the infection are attributed in part to the intestinal attaching and effacing attribute of the pathogen. This attribute requires expression of LEE (locus of enterocyte effacement) region genes of the STEC. Expression of LEE region genes and the resulting intestinal lesions are thought to be critical for intestinal colonization, production of clinically significant amounts of Shiga toxin in the intestine and in facilitating transport of Shiga toxin from the intestine into the blood where (in a proportion of patients) it causes the systemic vascular, renal and neurologic damage characteristic of the Hemolytic Uremia Syndrome.

Neonatal gnotobiotic or colostrum deprived piglets are the only animal model I am aware of wherein LEE region dependent colonization by *E. coli* O157:H7 results in attaching/effacing colonic lesions and diarrhea during a prodromal period, followed by transport of Shiga toxin to blood in sufficient quantities to cause systemic vascular damage, clinically apparent systemic manifestation of disease and death.

For the above reasons neonatal gnotobiotic or colostrum deprived pigs have a unique potential as a model to evaluate prophylactic or therapeutic approaches to human STEC infections.

Sincerely,

Harley W. Moon
Professor

Washington University Physicians
Washington University School of Medicine in St. Louis

Children's
HOSPITAL • ST. LOUIS

Research Units
Developmental Biology Unit
Cell and Molecular Biology Unit
Infection, Immunity & Inflammation Unit
Patient Oriented Research Unit

19 March 2003

To Whom It May Concern:

I am writing to render an unsolicited opinion regarding the prevention of systemic complications in *Escherichia coli* O157:H7 infection. I write from the position of a physician and researcher who has studied hundreds of children with this infection, a subset of whom who have developed hemolytic uremic syndrome (HUS).

Our data indicate that prothrombotic coagulation abnormalities are well underway early in illness (by day four of illness), well in advance of the development of HUS. Specifically, fibrinolysis is inhibited, and thrombin is being generated, and there is evidence for intravascular fibrin accretion in infected patients well before there is renal insufficiency, or renal tubular injuries (Chandler WL, et al. N Engl J Med 2002; 346:23).

Additionally, there is evidence in some infected patients for the fragmentation of circulating von Willebrand factor (Tsai HM, et al. Pediatr Res 2001; 49:653). Antibiotic administration has not been demonstrated to provide any benefit to infected children, and considerable data to suggest that such therapy actually increases the risk of developing HUS (Wong CS, et al. N Engl J Med 2000; 342:1930). Accordingly, it is my belief that if any toxin interdiction technologies are to work, they must be administered in the pre-symptomatic phase (following ingestion, prior to the first loose stool), or early in illness (as soon as diarrhea begins). The administration of such therapeutics after a culture is positive, would take place during a phase of illness when it is likely that the vascular insult would have already occurred.

To test whether such products given as soon as diarrhea begins are likely to be effective, an animal model is required which develops diarrhea well before the onset of systemic complications are apparent.

Yours sincerely,

Phillip I. Tarr, M.D.
Professor of Pediatrics and Microbiology

PIT/jfm

Washington University School of Medicine at
Washington University Medical Center,
McDonnell Pediatric Building
Campus Box 8208, 660 South Euclid Avenue

APPENDIX OF RELATED PROCEEDINGS

There is a related appeal in Serial No: 10/230,614 filed August 29, 2002, which directly affects, which would be directly affected by, or which may have a bearing on the Board's decision in this appeal. A decision was by the Board of Patent Appeals on September 26, 2005.

TABLE OF CONTENTS

PABST PATENT GROUP

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Date: November 11, 2005 **Total pages:** 59 including cover

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From: Patrea Pabst **Telephone:** 404-879-2151 **Telefax:** (404) 879-2160

Our Docket No. TUF 101 CIP
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Please call (404) 879-2150 if you did not receive all of the pages, or if they are illegible.

MESSAGE:

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appellant: Saul Tzipori, Ramaswamy Balakrishnan, and Arthur Donohue-Rolfe

Serial No.: 10/041,958 Art Unit: 1645

Filed: January 7, 2002 Examiner: Mark Navarro

For: *HUMAN NEUTRALIZING ANTIBODIES AGAINST HEMOLYTIC UREMIC SYNDROME*

Attachments:
Transmittal Form PTO/SB/21
Fee transmittal Form PTO/SB/17
Substitute Appeal Brief
Three (3) Declarations Under 37 C.F.R. 1.132
Three (3) References

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TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

Total Number of Pages in This Submission

Application Number	10/041,958
Filing Date	January 7, 2002
First Named Inventor	Saul Tziporl
Art Unit	1645
Examiner Name	Albert Mark Navarro
Attorney Docket Number	TUF 101 CIP

ENCLOSURES (Check all that apply)

- [x] Fee Transmittal Form
- [ ] Fee Attached
- [ ] Amendment/Reply
- [ ] After Final
- [ ] Affidavits/declaration(s)
- [ ] Extension of Time Request
- [ ] Express Abandonment Request
- [ ] Information Disclosure Statement
- [ ] Certified Copy of Priority Document(s)
- [ ] Reply to Missing Parts/ Incomplete Application
- [ ] Reply to Missing Parts under 37 CFR 1.52 or 1.53
- [ ] Drawing(s)
- [ ] Licensing-related Papers
- [ ] Petition
- [ ] Petition to Convert to a Provisional Application
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- [ ] After Allowance Communication to TC
- [ ] Appeal Communication to Board of Appeals and Interferences
- [x] Appeal Communication to TC (Appeal Notice, Brief, Reply Brief)
- [ ] Proprietary Information
- [ ] Status Letter
- [x] Other Enclosure(s) (please identify below):

Three (3) Declarations Under 37 C.F.R. 1.132
Three (3) References

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	Pabst Patent Group LLP		
Signature			
Printed name	Patrea L. Pabst		
Date	November 11, 2005	Reg. No.	31,284

CERTIFICATE OF TRANSMISSION/MAILING

I hereby certify that this correspondence is being facsimile transmitted to the USPTO or deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on the date shown below:

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Effective on 12/08/2004.
Fees pursuant to the Consolidated Appropriations Act, 2005 (H.R. 4818).

FEE TRANSMITTAL
For FY 2005

☑ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT	($) 0.00

Complete if Known	
Application Number	10/041,958
Filing Date	January 7, 2002
First Named Inventor	Saul Tzipori
Examiner Name	Albert Mark Navarro
Art Unit	1645
Attorney Docket No.	TUF 101 CIP

METHOD OF PAYMENT (check all that apply)

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FEE CALCULATION

1. BASIC FILING, SEARCH, AND EXAMINATION FEES

Application Type	FILING FEES Fee ($)	FILING FEES Small Entity Fee ($)	SEARCH FEES Fee ($)	SEARCH FEES Small Entity Fee ($)	EXAMINATION FEES Fee ($)	EXAMINATION FEES Small Entity Fee ($)	Fees Paid ($)
Utility	300	150	500	250	200	100	
Design	200	100	100	50	130	65	
Plant	200	100	300	150	160	80	
Reissue	300	150	500	250	600	300	
Provisional	200	100	0	0	0	0	

2. EXCESS CLAIM FEES

Fee Description	Fee ($)	Small Entity Fee ($)
Each claim over 20 or, for Reissues, each claim over 20 and more than in the original patent	50	25
Each independent claim over 3 or, for Reissues, each independent claim more than in the original patent	200	100
Multiple dependent claims	360	180

Total Claims	Extra Claims	Fee ($)	Fee Paid ($)	Multiple Dependent Claims Fee ($)	Multiple Dependent Claims Fee Paid ($)
11 - 20 or HP =	0 x		= 0.00		

HP = highest number of total claims paid for, if greater than 20

Indep. Claims	Extra Claims	Fee ($)	Fee Paid ($)
1 - 3 or HP =	0 x		= 0.00

HP = highest number of independent claims paid for, if greater than 3

3. APPLICATION SIZE FEE

If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

Total Sheets	Extra Sheets	Number of each additional 50 or fraction thereof	Fee ($)	Fee Paid ($)
- 100 =	/ 50 =	(round up to a whole number) x		=

4. OTHER FEE(S)

Fees Paid ($)

Non-English Specification, $130 fee (no small entity discount) _____

Other: _____

SUBMITTED BY

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